Clostridium perfringens-associated necrotic enteritis-like disease in coconut lorikeets (*Trichoglossus haematodus*)

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Abstract

Several outbreaks of necrotic enteritis-like disease in lorikeets, from which *C. perfringens* was consistently isolated, are described. All lorikeets had acute, segmental or multifocal fibrino-necrotizing inflammatory lesions in the small and/or the large intestine, with intralesional gram-positive rods. The gene encoding *C. perfringens* alpha toxin was detected by PCR on formalin-fixed, paraffin-embedded tissues (FFPE) in 20 out of 24 affected lorikeets (83%), but it was not amplified from samples of any of 10 control lorikeets ($p<0.0001$). The second most prevalent *C. perfringens* toxin gene detected was the beta toxin gene, which was found in FFPE from 7 out of 24 affected lorikeets (29%). The other toxin genes were detected inconsistently and in a relatively low number of samples. These cases seem to be associated with *C. perfringens*, although the specific type involved could not be determined.

Keywords: Alpha toxin, beta toxin, *Clostridium perfringens* type A, lorikeet, necrotizing enteritis, NetB toxin, Trichoglossus haematodus

Clostridium perfringens is an important cause of enteric diseases in animals. The *C. perfringens* species is currently divided into 7 types (A to G) on the basis of the presence of 6 major toxin genes, i.e.: alpha (*cpa*), beta (*cpb*), epsilon (*etx*), iota (*itx*), enterotoxin (*cpe*) and necrotic enteritis B-like toxin (*netb*).[14] Type G strains, encoding alpha- and NetB toxins, commonly cause necrotic enteritis (NE) in chickens and other poultry species.[7] Experimental and epidemiological evidence indicates that NetB toxin is essential for development of NE.[7,14] In addition, some authors suggest that *C. perfringens* type A (that encodes alpha toxin [CPA]) and C (that encodes CPA and beta [CPB] toxins) strains may also produce NE in poultry.[12] NE is a multifactorial disease, and coccidiosis, stress, energy and protein-rich diets are known predisposing factors in chickens.[16] Our knowledge about *C. perfringens*-associated enteritis in exotic birds is limited.[2,3,5,6,10,11] It has previously been suggested that this microorganism may cause an NE-like disease in both free-ranging and captive lorikeets.[6,10]

Between 2000 and 2018, 24 of the 67 (36%) lorikeets necropsied at the Institute of Animal Pathology of the University of Bern were diagnosed with NE-like disease, which represented the most frequent diagnosis. A consistent finding was the isolation of *C. perfringens* from the intestine of affected birds. We here describe the pathologic features of the disease and the results of PCR analyses for the detection of *C. perfringens* major toxin genes on DNA extracted from formalin-fixed, paraffin-embedded tissue of lorikeets.

The 24 coconut lorikeets (*Trichoglossus haematodus*) diagnosed with NE-like disease belonged to a zoological institution (A, n=21) or a private aviary (B, n=3), and had died spontaneously as part of five different outbreaks. Overall mortality in these outbreaks ranged from 29 to 60%. None of the outbreaks were associated with

introduction of new birds, or other identifiable causes of stress. The clinical histories included either sudden death or non-specific clinical signs such as apathy and separation from the flock shortly before death. Diseased lorikeets included juveniles (< 2 year-old, n=5), adults (≥2 year-old, n=13), and birds for which the age was not recorded (n=6); the age range was 7 months to 9 years. There were equal numbers of males and females. A full postmortem examination was performed and samples of small and large intestine, lung, heart and kidney in all cases, and of brain, pancreas, liver, spleen and skeletal muscle in most cases, were fixed by immersion in 10% buffered formalin, pH 7.2, for 24 to 72 hours. Tissues were routinely processed for histology and stained with hematoxylin and eosin (H&E); additional intestinal sections were also stained with Gram. The necropsy reports were reviewed.

Gross lesions were observed in 14 of the 24 lorikeets (58%), and were limited to the intestine. They consisted of segmental or multifocal, fairly well-demarcated transmural hyperemia and/or hemorrhage, focally or multifocally ulcerated mucosa, and multifocal to diffuse fibrinonecrotic membranes (Figure 1). Of the lorikeets that had gross abnormalities, six had lesions exclusively in the small intestine (43%), three (21%) had lesions only in the large intestine, and five (36%) had lesions in both the small and the large intestine. This is in contrast with previous reports in lorikeets and other exotic birds with NE-like disease, in which lesions were restricted to the small intestine.[6,10,15]

Despite gross lesions being reported in only 14 cases, all 24 lorikeets had histologically moderate or severe fibrino-necrotizing lesions in the intestine (Figure 2). The lesions were transmural in 13 (54%) cases, causing secondary peritonitis. The superficial epithelium and the lamina propria had extensive areas of necrosis. A fibrinonecrotic membrane composed of fibrin, viable and degenerate leukocytes,

blood and cellular debris covered the necrotic mucosa. Leukocyte infiltration of the mucosa and submucosa, mostly heterophilic with fewer lymphocytes, plasma cells and macrophages, was a prominent feature in 13 (54%) and mild in 11 (46%) birds. The inflammatory cells formed a band between the viable and the non-viable tissue. Myriad non-sporulated, gram-positive rods with morphology compatible with *Clostridium spp.* were observed within the fibrinonecrotic membrane, lamina propria and submucosa in all lorikeets (Figure 3). Fibrin thrombi were present in small arterioles and venules of the mucosa and/or submucosa in 88% of the cases. In addition, a few discrete foci of hepatocellular necrosis were randomly scattered throughout the hepatic parenchyma in two lorikeets (8%). No other significant microscopic lesions were observed in any bird. Overall, these lesions resembled acute *C. perfringens*-induced NE in poultry.

Bacteriological investigations were initiated soon after the necropsies for 16 of the 24 lorikeets (Table 1). Intestinal contents were incubated anaerobically for 24 h at 37 °C on membrane *C. perfringens* agar plates (mCP; Oxoid, Basel, Switzerland). Yellow, circular, opaque colonies typical for *C. perfringens* were obtained in 12 cases (75%). In seven of these cases, several of these colonies were pooled for DNA extraction and PCR detection of *C. perfringens* toxin genes as previously described.[1] These genes included *cpa*, *cpb*, *etx*, *iap*, *cpe* and *cpb2* (beta2 toxin). All samples were positive for *cpa. cpb2* was detected in samples of two lorikeets (Table 1). PCR was negative for the other toxin genes in all samples tested.

Causes of necrotizing enteritis in lorikeets include bacteria such as *Salmonella* spp.[20] and *C. colinum,*[13] and parasites such as coccidia.[17] Intestinal content from 16 animals was inoculated into enrichment in Muller-Kauffmann Tetrathionate-Novobiocin Broth (Oxoid, Ref: BO1224K) followed by subculture on Brilliance

Salmonella (Oxoid, Ref: PO5098A) and Brilliant Green Agar (Modified) (Oxoid, Ref: PO5033A). No *Salmonella* spp. were isolated in any of these 16 cases. No parasites were detected by using a combined sedimentation-flotation method with $ZnCl_2$ on intestinal contents in three lorikeets. No coccidia were detected on histological sections of any of the birds. Because no specific medium for *C. colinum* was used, a co-infection by this micro-organism cannot be ruled out.[13]

Because the above-mentioned *C. perfringens* toxinotyping PCR protocol was only performed on isolates from a subset of the lorikeets and did not include *netb*, we retrospectively evaluated the presence of this toxin gene and the other typing toxin genes[14] on DNA extracted from FFPE intestinal samples of all lorikeets affected by NE-like disease and from 10 control lorikeets without necrotizing intestinal lesions. Total DNA was extracted from three 10-µm thick paraffin sections using a QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Primers that were specific for short fragments of the main *C. perfringens* toxin genes were designed (Supplemental Table S1). DNA extracted from FFPE intestinal sections, from which the corresponding *C. perfringens* type had been isolated, was used as positive control. PCR amplicons were visualized in ethidium bromide-stained 1% agarose gels (Agarose SFP; Amresco, Solon, Ohio). Because all strains of *C. perfringens* produce CPA, the amplification of c*pa* was considered indicative of the presence of *C. perfringens* DNA in the sample. *Cpa* was detected in 20 of the 24 lorikeets affected by necrotizing intestinal lesions (83%) (Table 1), but in none of the 10 control lorikeets (p=0.0001, Chi-square with Yates' correction, GraphPad Prism, San Diego, California, USA). This is in agreement with previous data indicating that *C. perfringens* is uncommonly found in healthy lorikeets or other psittacines.[15] *C. perfringens* was, however, isolated from the intestine of two lorikeets negative for *cpa*

146 in the FFPE samples. Thus altogether, *C. perfringens* was detected in the intestine of
147 22/24 lorikeets (92%) affected by necrotizing intestinal lesions. In 10 (42%) of the
148 lorikeets with NE-like disease, *cpa* was the only toxin gene detected. The second
149 most prevalent *C. perfringens* toxin gene detected was *cpb* (29%), while the other
150 toxin genes were detected inconsistently and in a relatively low number of samples
151 (Table 1).

152 The role of CPA in intestinal diseases of animals has been suggested but never
153 definitively proven,[4,19] and there is no evidence to conclude from our results that CPA
154 was responsible for the lesions observed in our birds. We cannot however,
155 completely rule out a role for this toxin in the pathogenesis of the NE-like disease.
156 CPB is responsible for necrotizing enteritis in several animal species including
157 birds,[12,16] and the lesions described in these lorikeets were very similar to those
158 described in mammals and birds affected by this toxin.[12,18.] It is therefore possible
159 that CPB was responsible for the necrotizing intestinal lesions observed in at least
160 some of these lorikeets. This is in agreement with a previous report that identified
161 CPB in the intestine of lorikeets with necrotizing enteritis.[10] While NetB-producing
162 type G strains have been shown to play a major role in NE in chickens and other
163 poultry species,[7] *netB* was detected in only one lorikeet in this study.

164 In summary, our results suggest that, in lorikeets, a disease similar to the NE from
165 chickens is associated with the presence of *C. perfringens* in the intestine of the
166 lorikeets. *C. perfringens* type A was identified in 83% of lorikeets with NE-like
167 disease. We could not, however, conclusively demonstrate a role of a *C. perfringens*
168 toxin in the pathogenesis of this disease. It is possible that other yet unknown toxins
169 contributed to the necrotizing intestinal lesions in these lorikeets.[18] Similarly, CPA
170 was considered the key virulence factor for NE in broiler chickens for many years

until recent evidence demonstrated that NetB, and not CPA, is the main virulence factor of NE-producing type G strains.[7] NetF-positive type A strains may be involved in canine hemorrhagic gastroenteritis and equine necrotizing enteritis, although definitive evidence of the role of NetF in these diseases is lacking.[9] Moreover, several previously unknown toxin genes were identified in isolates from turkeys, [8] indicating a much more diverse picture of pathogenic *C. perfringens* type A isolates. However, most studies (including ours) lack consistent isolation and full characterization of *C. perfringens* isolates from the intestine of diseased animals. Fulfillment of Koch's postulates is also lacking. Therefore, conclusions on a causal relationship of particular pathogenic strains of *C. perfringens* type A with NE-like disease in lorikeets or other exotic birds cannot be drawn. To investigate the causal relationships of different *C. perfringens* with NE-like disease in animals, whole-genome sequencing of *C. perfringens* isolates should be considered, along with experimental work to fulfill Koch's postulates.

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Figure Legends

Figure 1. Necrotizing enteritis, small intestine, lorikeet. There is multifocal reddening of the intestinal wall, which corresponds to necrotizing enteritis visible from the serosal surface. The intestinal serosa has multifocal white areas, which correspond to areas of transmural inflammation and necrosis (peritonitis, arrow). Inset: The mucosa is diffusely necrotic.

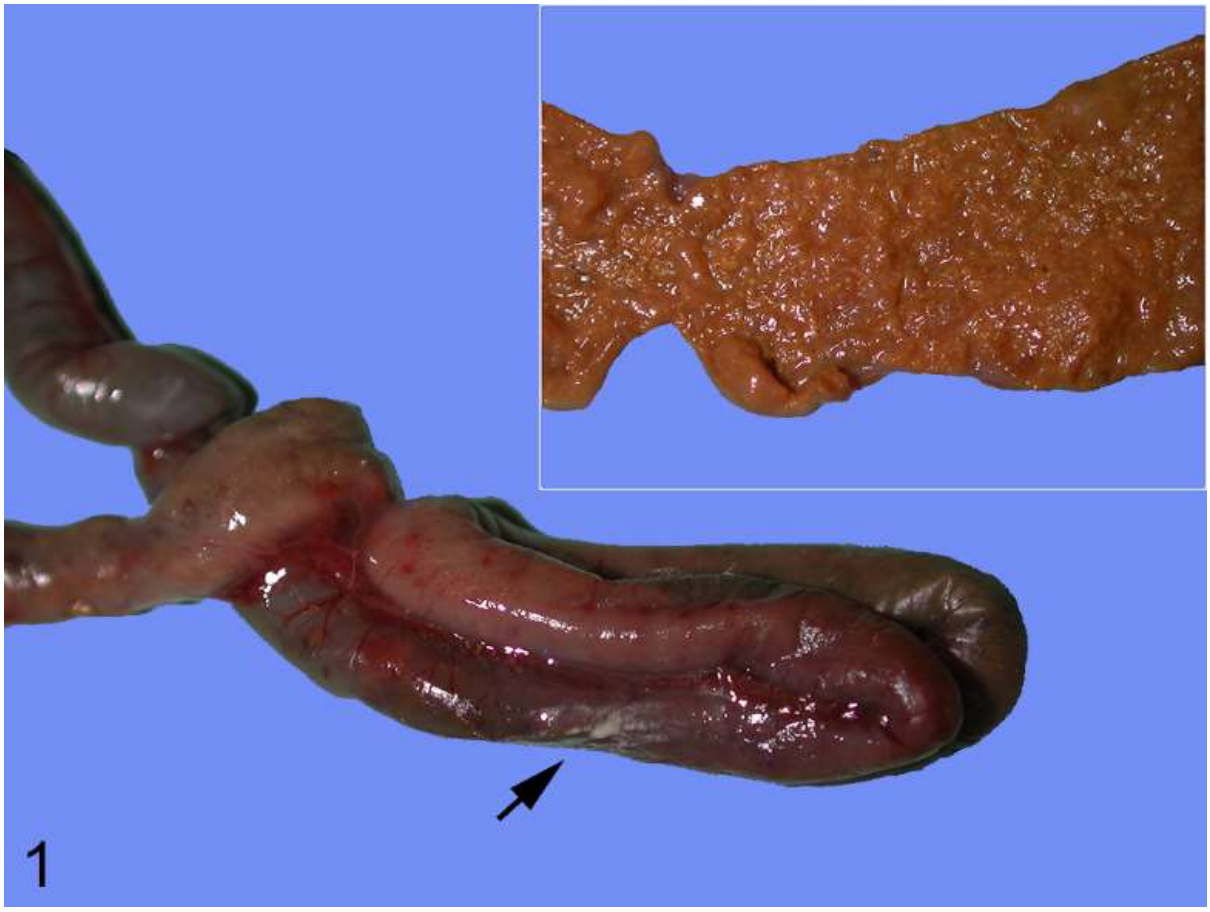
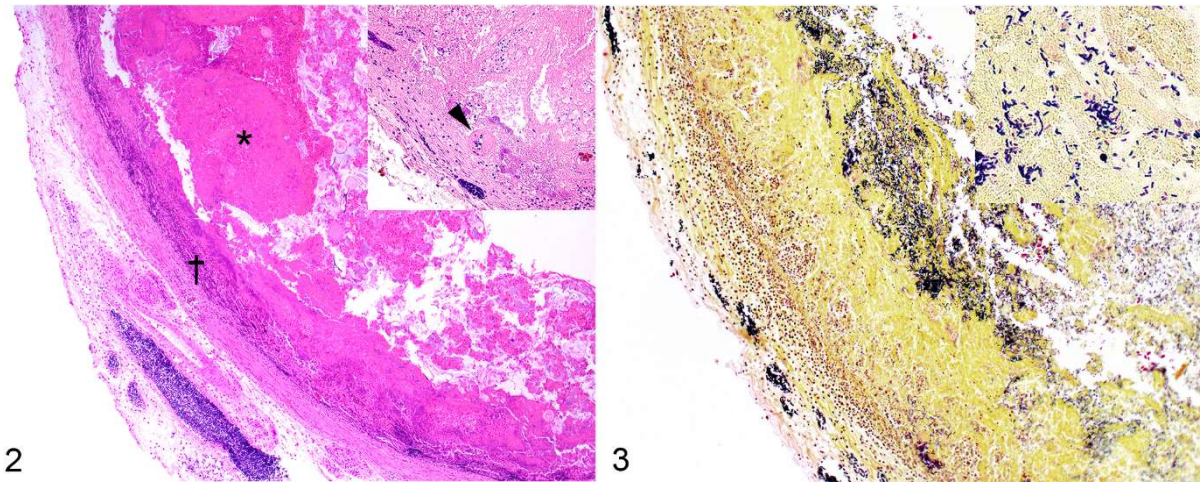


Figure 2. Transmural fibrinonecrotizing enteritis, small intestine, lorikeet. The mucosa is diffusely necrotic and covered by a fibrinonecrotic membrane (asterisk). There is loss of the mucosal/submucosal boundary and transmural infiltration with inflammatory cells (dagger). Inset: The mucosa contains a fibrin thrombus within a small vessel (arrowhead).

Figure 3. Small intestine, lorikeet. Abundant gram-positive rods are present within the necrotic mucosa and the intestinal lumen. Inset: Bacillary morphology of the gram-positive Clostridial-like bacteria in the necrotic mucosa.



294 **Table 1**. Results of *Clostridium perfringens* culture and PCR typing in 24 lorikeets with necrotic enteritis-like disease.

Outbreak Number and Origin (A or B)	**Animal Number**	***Clostridium perfringens* isolation**	**PCR on FFPE intestine and on isolates (in brackets)[a]**							**Inferred Possible Toxinotypes[b]**
			cpa (alpha toxin)	*cpb* (beta toxin)	*etx* (epsilon toxin)	*iap* (iota toxin)	*cpe* (CPE)	*netB* (NetB)	*cpb2* (beta2 toxin)	
1 (A)	1	-	+	-	-	-	-	-	NP	A
1 (A)	2	NP	+	+	-	-	-	-	NP	A, C
1 (A)	3	+	+	-	-	-	+	+	NP	A, F, G
1 (A)	4	-	+	-	-	-	-	-	NP	A
1 (A)	5	NP	-	-	-	-	-	-	NP	-
1 (A)	6	-	-	-	-	-	-	-	NP	-
1(A)	7	+	-	-	-	-	-	-	NP	-
1 (A)	8	+	-	-	-	-	-	-	NP	-
2 (B)	9	+	+	-	-	-	-	-	NP	A
2 (B)	10	-	+	-	-	-	-	-	NP	A
3 (B)	11	NP	+	+	+	-	-	-	NP	A, C, D
4 (A)	12	+	+ (+)	**+ (-)**	- (-)	- (-)	- (-)	-	NP (-)	A, C
4 (A)	13	NP	+	-	-	-	-	-	NP	A
4 (A)	14	NP	+	-	-	-	-	-	NP	A
4 (A)	15	NP	+	-	-	-	-	-	NP	A
4 (A)	16	+	+ (+)	- (-)	- (-)	- (-)	- (-)	-	NP (-)	A
4 (A)	17	NP	+	+	+	-	+	-	NP	A, B, C, D, F
4 (A)	18	+	+	+	+	-	+	-	NP	A, B, C, D, F
4 (A)	19	NP	+	+	+	-	-	-	NP	A, C, D
4 (A)	20	+	+ (+)	- (-)	- (-)	- (-)	**+ (-)**	-	NP (-)	A, F
5 (A)	21	+	+ (+)	- (-)	- (-)	- (-)	- (-)	-	NP (-)	A
5 (A)	22	+	+ (+)	- (-)	- (-)	- (-)	- (-)	-	NP (-)	A
5 (A)	23	+	+ (+)	-	-	+	-	-	NP (+)	A, E
5 (A)	24	+	+ (+)	+	+	+	+	-	NP (+)	A, B, C, D, E, F
TOTAL [Percentage]		12/16 [75%]	20/24 [83%]	7/24 [29%]	5/24 [21%]	2/24 [8%]	4/24 [17%]	1/24 [4%]	2/7 [29%]	

295 Abbreviations: CPE, *Clostridium perfringens* Enterotoxin; FFPE, formalin-fixed, paraffin-embedded; NetB, necrotic enteritis B-like; NP,
296 not performed.

297 [a]PCR testing was done on FFPE intestine from all lorikeets, and from the bacterial isolates in 7 of the animals. PCR results are given
298 as + (positive) and – (negative). PCR results on bacterial isolates is indicated in brackets. Two discrepant PCR results are highlighted
299 in bold.

300 [b]The *C. perfringens* toxinotypes possibly involved in each lorikeet are listed.

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Supplemental Table 2. Primers used for detection of the genes encoding the typing toxins of *C. perfringens* in formalin-fixed, paraffin-embedded sections of intestinal tissue.

Primer name	Sequence (5'-3')	Target gene	Product size (bp)
CPAF	AAGGCGCTTATTTGTGCCG	*cpa* (alpha toxin)	101
CPAR	GCATGAGTTCCTGTTCCATCA		
CPBF	GCGAATATGCTGAATCATCTA	*cpb* (beta toxin)	196
CPBR	GCAGGAACATTAGTATATCTTC		
ETXF	GAAGTGAATGGGGAGAGATACCTA	*etx* (epsilon toxin)	160
ETXR	ATTAACTCATCTCCCATAACTGCAC		
ITXF	TTGTATATAGAAGGTCTGGTCCAC	*iap* (iota toxin)	127
ITXR	GGGTATGTTATTACTTTTCCTTCCC		
CPEF	TGGATATTAGGGGAACCCTCAG	*cpe* (enterotoxin)	227
CPER	TTTGGACCAGCAGTTGTAGATA		
NetBF	ATCCTCATTCTGATAAGAAAACTGC	*netB*	250
NetBR	TTTCCTTCAACAGATATATTACCGC		

PCR performed in a total volume of 25 µL containing 0.5 µL of each primer (0.5 µM), 5 µL of extracted DNA, 7 µL of nuclease-free water and 12 µL of PCR Master Mix 2X Promega (Madison, Wisconsin). Thermocycler profiles were as follows: 95°C for 10 minutes, 35 cycles of 95°C for 35 seconds, 50°C for 35 seconds, and 72°C for 35 seconds, and a final extension step at 72°C for seven minutes.